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STUDIES ON THE IMMUNOCHEMICAL TECHNIQUES FOR DETECTION OF SELECTED FUNGAL AND DINOFLAGELLATE TOXINS

Annual Report

August 31, 1985

F. S. Chu, Ph.D.

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The progress during the third and 4th years of our contract (Aug. 1, 1983 to Aug. 31, 1985) are summarized as follows: (i) Methods for the production of antibodies in rabbits against diacetoxyscirpenol (DAS), deoxyverrucarol (DOVE), acetyl-deoxynivalenol (Ac-DON) and T-2 toxin metabolites including 3'-OH-T-2, H-T-2, T-2 tetraol tetra-acetate were developed. These antibodies were highly specific to the respective trichothecenes or their metabolites that have been used in the conjugation. (ii) A number of tritiated trichothecene mycotoxins of high specific radioactivity were prepared in our laboratory. These labelled toxins have been used as marker ligands for RIA of these mycotoxins as well as for metabolic studies. (iii) A new improved method for the production of antibodies against T-2 toxin and DAS in rabbits was developed. More stable protein conjugates of carboxyethyl derivatives (CMO) of these toxins were used in the immunization. (iv) With the

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(19). availibility of different antibodies, sensitive ~~10.1-0.5 ng/assay~~ and specific RIAs for these mycotoxins were developed. A direct competitive ELISA for DAS with a detection limit of 25 pg per assay was developed. However, because of interferring substances in the saeple matrix, the minimal detection levels for DAS in wheat and corn by RIA and ELISA are found to be 25 and 50 ppb, respectively. (v) An indirect ELISA with detection limits of 25 pg /assay for analysis of saxitoxin (STX) was developed. The detection limit for analysis of STX in mussels and clams by this method was found to be around 50-100 ppb without sample treatment. Saxitoxin antibody raised after immunizing rabbits with STX-HCHO-BSA showed high specificity to STX. Cross-reaction of this antibody with decarbamoyl-STX (56%) and neo-STX (16%) was observed. (vi) The ability of to produce T-2 toxin and DAS by 18 fusaria was studied by RIA. Among 13 F. sporotrichioides tested, all but one were found to be T-2 toxin or/and DAS producers. The amount of toxin(s) produced by each species varied considerably with species and incubation temperatures. Three F. graminearum and 2 F. chlamydosporum cultures did not produce any T-2 toxin and DAS. (vii) Limited efforts for production of monoclonal antibodies for STX and T-2 toxin were made; but no stable clones were obtained. ~~(viii)~~ The metabolism of T-2 toxin in vitro was found to be affected by esterase inhibitors greatly. Thus, an efficient method for the preparation of large quantities of ~~3'-OH~~-T2 toxin, 3'-OH-HT-2, 3'-OH-acetyl-T-2 and 3'-OH-T-2 triol was developed. This method involves the incubation of T-2 toxin with swine liver (phenobarbital induced) S-9 preparation in the presence of $NADP^+$ regeneration system and an esterase inhibitor. ~~(ix)~~ Routine production of antibodies against T-2 toxin and DAS continued with the new T-2 toxin and DAS derivatives. Some immunochemical reagents were made and delivered to the USAMRIID.

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FOREWARD

The following is the third annual report of the work performed under contract No. DAMD17-82-C-2021, during the period of Aug. 1, 1983 to Aug. 31, 1985. The work was carried out at the Food Research Institute of the University of Wisconsin-Madison, under the direction of the principal investigator, Dr. F. S. Chu and co-principal investigator, Dr. E. J. Schantz. The contract officer is Dr. Robert W. Wannemacher, Jr.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

ABSTRACT

The progress during the third and 4th years of our contract (Aug. 1, 1983 to Aug. 31, 1985) are summarized as follows: (i) Methods for the production of antibodies in rabbits against diacetoxyscirpenol (DAS), deoxyverrucarol (DOVE), acetyl-deoxynivalenol (Ac-DON) and T-2 toxin metabolites including 3'-OH-T-2, H-T-2, T-2 tetraol tetra-acetate were developed. These antibodies were highly specific to the respective trichothecenes or their metabolites that have been used in the conjugation. (ii) A number of tritiated trichothecene mycotoxins of high specific radioactivity were prepared in our laboratory. These labelled toxins have been used as marker ligands for RIA of these mycotoxins as well as for metabolic studies. (iii) A new improved method for the production of antibodies against T-2 toxin and DAS in rabbits was developed. More stable protein conjugates of carboxymethyl derivatives (CMO) of these toxins were used in the immunization. (iv) With the availibility of different antibodies, sensitive (0.1-0.5 ng/assay) and specific RIAs for these mycotoxins were developed. A direct competitive ELISA for DAS with a detection limit of 25 pg per assay was developed. However, because of interferring substances in the sample matrix, the minimal detection levels for DAS in wheat and corn by RIA and ELISA are found to be 25 and 50 ppb, respectively. (v) An indirect ELISA with detection limits of 25 pg /assay for analysis of saxitoxin (STX) was developed. The detection limit for analysis of STX in mussels and clams by this method was found to be around 50-100 ppb without sample treatment. Saxitoxin antibody raised after immunizing rabbits with STX-HCHO-BSA showed high specificity to STX. Cross-reaction of this antibody with decarbamoyl-STX (56%) and neo-STX (16%) was observed. (vi) The ability of to produce T-2 toxin and DAS by 18 fusaria was studied by RIA. Among 13 F. sporotrichioides tested, all but one were found to be T-2 toxin or/and DAS producers. The amount of toxin(s) produced by each species varied considerably with species and incubation temperatures. Three F. graminearum and 2 F. chlamydosporum cultures did not produce any T-2 toxin and DAS. (vii) Limited efforts for production of monoclonal antibodies for STX and T-2 toxin were made; but no stable clones were obtained. (viii) The metabolism of T-2 toxin *in vitro* was found to be affected by esterase inhibitors greatly. Thus, an efficient method for the preparation of large quantities of 3'-OH-T2 toxin, 3'-OH-HT-2, 3'-OH-acetyl-T-2 and 3'-OH-T-2 triol was developed. This method involves the incubation of T-2 toxin with swine liver (phenobarbital induced) S-9 preparation in the presence of $NADP^+$ regeneration system and an esterase inhibitor. (ix) Routine production of antibodies against T-2 toxin and DAS continued with the new T-2 toxin and DAS derivatives. Some immunochemical reagents were made and delivered to the USAMRIID.

I. INTRODUCTION

Since the last annual report, our efforts have been focused on the development of methods for the production of antibodies against several important trichothecenes. Efforts for the production of antibody against metabolites of T-2 toxin and diacetoxyscirpenol (DAS) have also been made. In addition, we have developed a new method to prepare stable T-2 and DAS-protein conjugates. Using different approaches, we have successfully produced useful antibodies with different specificities against T-2 toxin, HT-2, 3'-OH-T-2, T-2 tetraol tetra-acetate, DAS, deoxyverrucarol (DOVE), and acety-deoxynivalenol (DON) and saxitoxin. Properties of such antibodies were investigated in detail. We have also developed an efficient method for the production of 3'-hydroxyl derivatives of T-2 and T-2 toxin metabolites, as well as effective methods for the preparation of various radioactive trichothecenes. With the availability of such antibodies, studies to optimize different immunoassays, including both radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) of such toxins in different matrices have also been carried. Studies for the application of such methods including those such as the use of RIA for monitoring of T-2 and DAS produced by selected Fusaria and the use of antibodies for immunohistochemical stain of DAS in tissues were performed. In this report, the essence of such studies are presented.

II. WORK PERFORMED AFTER LAST ANNUAL REPORT

I. STUDIES ON T-2 TOXIN:

(A) Production of antibodies against T-2 toxin:

(1) Long term immunization of rabbits with T-2 hemisuccinate (HS)-BSA and T-hemiglutarate (HG)-BSA: Routine production of antibodies against T-2 toxin continued. Seven rabbits continued to be boosted with either T-2-HS-BSA (4 rabbits) or T-2-HG-BSA (3 rabbits) conjugates every 5 weeks. The maximal antibody titers for these rabbits after each boosting were around 8,000-14,000. For DAS, six rabbits were boosted on a monthly basis. The titers were in the range 100-2,200.

(2) Production and characterization of antibodies after immunizing rabbits with CMO-T-2-BSA conjugates: A new, improved approach for the production of antibodies against T-2 toxin was developed. The method involves the use of immunogens which were prepared by conjugating O-carboxylmethoxyl oxime (CMO) derivatives of T-2 toxin to bovine serum albumin (BSA). Two isomers (isomer "a" and "b") of CMO-T-2 toxin were tested. Antibodies against both isomers were demonstrated as early as 4 weeks after immunization. Conjugate of "a" isomer of CMO-T-2-BSA was found to be a better immunogen than the b isomer and highest titers (6,000) were reached 14 weeks after immunization and one booster injection. Antibody titers for rabbits immunized with the "b" isomer of CMO-T-2 toxin never reached more than 2,000. The specificity of antibodies obtained from rabbits after immunizing with CMO-T-2-BSA was similar to that of hemisuccinate-T-2-BSA. The anti-b-T-2 antibodies had slightly higher cross-reactivity with HT-2 toxin as compared with the

antibody obtained from rabbits immunized with the conjugate of the "a" isomer. The relative cross-reactivity of anti-"a"-CMO-T-2 antibody with T-2, acetyl-T-2, HT-2, T-2-triol, 3'-OH-T-2 and T-2 tetraol were found to be 1, 4.5, 5.7, 250, 500 and 3000, respectively. The relative cross-reactivity of anti-b-T-2 antibody with T-2, acetyl-T-2, HT-2 and T-2 triol were found to be 1, 2, 3 and 488, respectively. The affinities of anti-"a"-CMO-T-2 toxin and anti-"b"-CMO-T-2 toxin, with T-2 toxin were found to be 5.07×10^{9} and 7.87×10^{9}, respectively. Details for this study as well as a discussion on the role of side chains groups for eliciting antibodies against group-A types of trichothecenes are presented in a manuscript (publication no. II-1).

(B) Studies on 3'-OH and related T-2 toxins:

(1) In vitro preparation of different 3'-OH derivatives of T-2 toxin: A new approach for the production of large quantities of 3'-OH-T-2 toxin was developed in our laboratory. Our approach included incubation of T-2 toxin with S-9 fraction isolated from livers of rats and pigs which had been treated with phenobarbital, together with $NADP^{+}$ regenerating system and diethyl-p-nitrophenol (DENP), an esterase inhibitor. T-2 toxin metabolites were isolated from the incubation mixture by an Amberlite XAD-2 column and TLC. We found that, DENP was capable of blocking the hydrolytic pathway completely at a concentration of 10^{-4} M. Instead of converting of T-2 toxin to HT-2 toxin, the metabolism shifted to the hydroxylation route. As high as 83% of the T-2 toxin was converted to 3'-OH-T-2 toxin. Using the same approach, 3'-OH-HT-2 and 3'-OH-acetyl-T-2 toxin were prepared. The conversion yield of T-2 toxin, acetyl T-2 toxin, HT-2 toxin and T-2 triol to their respective 3'-hydroxyl derivatives were 82%, 73%, 72% and 75%, respectively. Therefore, this approach can be used for the preparation of large quantities of the hydroxyl derivatives of T-2 and related toxins. The chemical structures of these compounds were confirmed by MS and NMR. Since 3'OH-T-2 toxin is as toxic as T-2 toxin, present results implicate that the toxicity of T-2 toxin could be altered when esterase blockers are present. Details of this study are presented in a manuscript published in AEM (publication No. I-9).

(2) Binding of 3'-OH T-2 toxin with anti-T-2 HS antibody: With the availability of pure 3'-OH T-2 toxin, the cross-reactivity of T-2-HS antisera with this new metabolite was determined. The cross-reactivity of T-2 antisera with this metabolite was 200 times less than T-2 toxin.

(3) Production and characterization of antibody against 3'-OH-T-2 toxin: Since antibody raised against T-2 toxin cross-reacted poorly with 3'-OH-T-2 toxin, a new immunogen was prepared by conjugation of hemisuccinate (HS) of 3'-OH-T-2 toxin to bovine serum albumin (BSA). Antibodies against 3'-OH-T-2 toxin were demonstrated by a radioimmunoassay 10 weeks after immunizing rabbits with this new immunogen using tritiated 3'-OH-T-2 toxin as the test ligand. Highest titers (6,000) were obtained 17 weeks after immunization and two booster injections. The antibodies showed good cross-reactivity with T-2 toxin, acetyl-T-2 toxin, 3'-OH-acetyl-T-2 toxin. The relative cross-reactivity of this antibody with 3'-OH-T-2, acetyl-T-2, T-2, 3'-OH-acetyl-T-2, 3'-OH-T-2 HS, T-2 isomer, HT-2, and 3'-OH-HT-2 were found to be 1, 3, 4,

5, 15, 30, 45 and 175, respectively. No cross-reaction was found when 3'-OH-T-2 triol, T-2-triol, T-2-tetraol, DAS and DON at a concentration of 1 ug per assay was tested. The detection limit for 3'-OH-T-2 toxin by the RIA was found to be around 0.1 ng per assay. Details of this study are presented in a manuscript (publication no. II-2).

(C) Production and characterization of antibodies against T-2 toxin metabolites other than 3'-OH-T-2 toxin:

(1) Production and characterization of antibody against HT-2. Conjugation of HT-2 to BSA was achieved by coupling b isomer of CMO-HT-2 to the protein in the presence of a water soluble carbodiimide. Three rabbits of each group were immunized with the immunogen. Antibodies against HT-2 toxin were demonstrated in the rabbits as early as 4 weeks after immunization and the titers are continuing to increase after booster injection as determined by the binding of tritiated HT-2 toxin. The specificity of anti-CMO-HT-2 antibody was determined by a competitive RIA using tritiated HT-2 as the marker ligand. The results show that the antibody is specific for HT-2, but also has good cross-reaction with T-2 toxin. The concentrations causing 50% inhibition of binding of tritiated HT-2 to this antibody by unlabeled HT-2, T-2, T-2 isomer, acetyl-T-2, 3'-OH-HT-2, 3'-OH-T-2, and T-2 triol were found to be 0.62, 2.5, 6.2, 18.6, 248, 420, and 530 ng per assay, respectively. Thus, the anti-HT-2 toxin antibodies showed good cross-reaction with T-2 toxin, but with less cross-reactivity with T-2 triol, T-2 tetraol and 3'-OH-T-2 toxin. Using tritiated HT-2 as the test ligand, the detection limit for HT-2 assays by RIA was found to be around 0.2 ng per assay. An indirect ELISA which has a detection limit of 5 pg per assay was also established for the HT-2 toxin.

(2) Production of antibodies against T-2 tetraol and T-2 tetraol tetra-acetate: Tritiated T-2 tetraol was prepared from T-2 toxin after hydrolysis with base and was subsequently purified by preparative TLC according to Wei, et. al. (Biochem. Biophys. Res. Comm. 45: 396, 1971). CMO-T-2 tetraol was prepared from CMO-T-2 (both "a" and "b" isomers) the same way. CMO-T-2 tetraol tetra-acetate of both isomers were prepared by acetylation of the CMO-T-2 tetraol isomers. The "b"-CMO-T-2 tetraol and both "a" and "b" isomers of CMO-T-2 tetraol tetra-acetate were conjugated to BSA for subsequente immunization (12, 21 and 19 moles/mole of BSA for "a"-CMO-T2-401, "a"-CMO-T-2-TA and "b"-CMO-T-2-TA, respectively.

Among these three immunogens tested, "b"-CMO-T-2-TA was found to be the best immunogen. Antibody titers against T-2 tetraol have not been demonstrated in rabbits which had been immunized with T-2 tetraol-BSA 20 weeks after initial immunization and 3 booster injections. Antibody titers for "b"-CMO-T-2-TA were low and only 100 after 12 weeks and 2 booster injections. However, rabbits immunized with "b"-CMO-T-2 -tetra-acetate-BSA conjugates showed good antibody titers (titers around 2500, 11 weeks after immunization and 1 booster injections). Experiments designed to characterize this new antibody as well as to use this antibody for analysis of T-2 tetraol are underway.

II. STUDIES ON DIACETOXYSCIRPENOL (DAS):

(A) Production and characterization of antibodies against DAS by immunizing rabbits with HS-DAS-BSA conjugate:

We have previously reported (second annual report) that antibody against DAS was obtained from rabbits after immunizing with DAS-HS-BSA. Efforts to characterize the antibody were made in the past year. In a competative RIA, unlabeled DAS, deoxynivalenol (DON), deoxyverrucarol (DOVE), T-2 toxin, 4-monoacetylscirpenol (4-MAS), 15-MAS and scirpenol triol were tested for their ability to displace the radioactive DAS from the antiserum. All these compounds did not show appreciable cross-reaction with the antiserum except 4-MAS which was capable of displacing the binding of 3H-DAS with the antiserum. However, 4-MAS was about 80 times less effective than non-radioactive DAS. Details of this study are presented in publication no. I-5).

(B) Preparation and characterization of antibody against DAS by immunizing with "b"-CMO-DAS-BSA conjugate:

Antibodies against "b"-CMO-DAS was prepared using the the same approach as those for T-2 toxin. However, these antibodies showed a high degree of cross-reactivity with monoacetoxyscirpenols. The relative cross-reactivity of the antisera for DAS, 4-MAS, 15-MAS, acetyl-DON, T-2 toxin, acetyl-T-2 toxin and neosolaniol were found to be 1, 4, 5, 76, 107, 147, and 265, respectively. The affinity constants of anti-"b"-DAS to DAS, 4-MAS and 15 MAS were found to be 4.89, 1.09, 0.87 x 10^7 liters/mole, respectively. On the other hand, the affinity constants of anti-DAS-HS with DAS, 4-MAS and 15-MAS were found to be 4.23 x 10^9, 1.15 X 10^6 and 1.44 X 10^6 liters/mole, respectively. Because of their cross-reactivity, unlike the anti-HS-DAS-BSA antibodies, the anti-"b"-CMO-DAS antibodies could be used for immunoassays of MAS. Details of this study are presented in publication no. II-1.

(C) Development of immunoassay for DAS:

With the availability of a specific antiserum against DAS, we also tested a direct ELISA for DAS. The diluted antiserum was coated to the plate by the glutaraldehyde method. Both DAS-hemiglutarate (HS)-horseradish-peroxidase (HRP) and DAS-hemisuccinate (HS)-HRP conjugates were tested for their ability to bind with the antiserum. DAS-HS-HRP, which was found more effective than that DAS-HS-HRP preparation, was used in the subsequent studies. The results obtained from competitive ELISA studies were found to be similar to those obtained from RIA. The ELISA permits detection as low as 25 pg of DAS in each assay (1 ng/ml and 25 ul used in each assay).

Protocols for both RIA and ELISA for DAS in corn and wheat were developed. The detection limits for DAS in corn were found to be 25 and 50 ppb by RIA and ELISA, respectively. We found that considerable interfering substances were removed after SepPak treatment. The recovery

of DAS added to the wheat and corn samples between 5-100 ppb by ELISA were found to be between 60-90%.

(D) Preparation of DAS metabolites:

Efforts for the production of large quantities of DAS metabolites continued. In an in vitro study, S-9 fractions of monkey liver were incubated with DAS in the presence or absence of a NADPH regeneration system. We found that the major metabolite was 15-MAS. 4-MAS was also detected.

III. PRODUCTION OF T-2 TOXIN AND DAS BY DIFFERENT FUSARIA AS ANALYZED BY RIA:

(A) Effect of temperature on the production of T-2 toxin and DAS:

Production of T-2 toxin and DAS by two Fusarium sporotrichioides species (one previously identified as F. tricinctum) at four different temperatures (7, 15 C, 24 and 28C) in a glucose-soya meal-corn steep liquor medium absorbed in vermiculite was studied. After incubation for appropriate time, the toxins were extracted from the culture with methanol, subjected to a Sep-Pak cartridge treatment, and then were analyzed by RIA. Fusarium tricinctum was found to be primarily a T-2 toxin producer as little or no DAS was found in the culture medium. On the contrary, F. sporotrichioides produce both toxins; the amount of DAS was three times higher than T-2 toxin. Higher temperature (28C) was more favorable for DAS production whereas in contrast, a higher yield of T-2 toxin was obtained at lower temperature (15C). Maximum toxin yield was obtained 20-30 days after incubation at both temperatures. At 28 C, large amounts of DAS (600-700 mg/liter) were produced by F. sporotrichioides in each culture whereas less than 200 mg of T-2 was obtained after 25 days of incubation. At 15 C, the DAS and T-2 produced by this fungi were found to be around 210 mg and 234 mg per liter, respectively. For F. tricinctum, between 740-900 mg and 630-740 mg of T-2 toxin per liter were produced after 20-30 days of incubation at 15 C and 24 C, respectively. Less than 11 mg/liter of DAS was produced by F. tricinctum under these conditions. Our results are consistent with earlier data which was obtained by GLC analysis. In addition, our results also show the potential health hazard of some Fusaria species that produce more than one type of toxic trichothecenes under field conditions.

(B) Studies on the production of T-2 toxin and DAS by different strains of Fusaria:

The production of T-2 toxin and DAS by 18 Fusaria in above medium at 15C and 24C over a period of 35 days was studied. The cultures were supplied by Dr. Nelson of University of Penn. Among 13 strains of F. sporotrichioides tested, 4 were found to be high T-2 toxin producers (greater than 700 mg of T-2/liter at 15C and 200 mg/L DAS at 24C), 2 were high DAS producers (700-900 mg/L DAS at 24C; 200 mg/L T-2 at 15C); 3 strains produced both toxins in good yield (300-600 mg of both toxin at 24 or 15C), 3 were identified as low toxin producers (less than 300 mg/liter) and one did not produce T-2 toxin and DAS. T-2 toxin and DAS

were not detected in the medium inoculated with 3 strains of F. graminearum and two F. chlamydosporum.

IV. PRODUCTION OF ANTIBODIES AGAINST VERRUCARRIN A AND DEOXYVERRUCAROL:

(A) Production and characterization of antibody against deoxyverrucarol (DOVE):

The antibody against DOVE was produced by immunizing rabbits with DOVE-HS-BSA conjugate. The antibodies bound with either tritiated DOVE or diacetoxyscirpenol (DAS), but not with tritiated T-2 toxin. The affinity of antibodies with DOVE was found to be much higher than DAS. When ^{3}H-DOVE was used as a marking ligand in the competitive RIA, the concentrations causing 50% inhibition of binding radioactivities by the unlabeled DOVE, verrucarol, verrucarin A, and 4-MAS were found to be 0.32, 1070, 9500 and 10,000 ng per assay, respectively. T-2 toxin, 15-MAS, and deoxynivalenol gave less than 20% inhibition at 10 ug per assay tested. However, when ^{3}H-DAS was used as the marking ligand, the concentrations causing 50% inhibition by DOVE, DAS and verrucarol were found to be in the 50-60 ng per assay range. The antibodies thus are highly specific to DOVE rather than a common trichothecene backbone. The possible use of this antiserum for assay of macrocylic trichothecenes is discussed. Details of this study are presented in publication no. I-6.

(B) Production of antibody against verrucarin A:

An attempt for the production of antibody against verrucarin A was made during the last few months. Verrucarin A was conjugated to BSA after converting to its hemisuccinate. A tritiated verrucarin A was prepared by reduction of 2-dehyroverrucarin A with tritiated $NaBH_4$. However, no antibody titer was demonstrated 12 weeks after immunization. We plan to synthesize a hemiglutarate verrucarin A and then conjugate to protein for immunization.

V. STUDIES ON DEOXYNIVALENOL (DON):

(A) Preparation of different DON derivatives:

Since our last annual report, several new DON derivatives, including HS and HG of DON, 15-aldehyde DON and HS of 7,8 dihydorxycalonectrin (DHC), were prepared. DON-3-HS and DON-3-HG were prepared according to the following three steps: (a) first to prepare a benzylidene(BZ)-DON in which the hydroxyl groups at 7 and 15 positions were protected; (b) the BZ-DON was then reacted with succinic or glutaric anhydride; and (c) finally the benzylidene groups were removed from the 7 and 15 positions. The yield of recovery during these steps were low, and only 33-50% of DON-3-HG and DON-3-HS were obtained from the BZ-DON. 15-aldehyde-DON wasprepared by mild oxidation and characterized by mass spectral analyses (C.I. and E. I.).

(B) Preparation of highly specific radioactive tritiated DON and acetyl-DON:

A radioactive DON was prepared by oxidation of the CH OH at the 15 position to 15 aldehyde-DON and then reduction back to the CH_2OH again with highly specific tritiated $NaBH_4$ (sp. activity 79.1 Ci/mmol) under very mild conditions (9% of tritium to DON and 2% of tritium to 8-OH-DON). Tritiated acetyl-DON was prepared by acetylation of H-DON with acetic acid anhydride in the presence of pyridine and then purified by TLC.

(C) Conjugation of DON-3-HS, DON-3-HG and 15-aldehyde-DON to BSA and subsequent immunizations:

A mixed anhydride method was used for the conjugation of DON-3-HS or DON-3-HG to bovine serum albumin (BSA) and 23 moles of DON-HG and 13 moles of DON-HS were conjugated to one mole of BSA. 15-aldehyde-DON was conjugated to BSA for immunization by reductive alkiyation reaction. Two groups of rabbits were immunized with either BSA-DON-HS (6 rabbits) or BSA-DON-HG (3 rabbits). However, none of the rabbits were shown to have high antibody titers against DON when the highly specific radioactive DON was used as the ligand (less than 50% of binding of 6,000 dpm of ^{3}H-DON with 0.1 ml of antiserum) in the radioimmunoassay 30 weeks afer immunization, with 5 booster injections. Rabbits immunized with 15-aldehyde-DON-BSA for more than 35 weeks with 3 booster injections also did not show any antibody against DON.

(D) Production and characterization of antibody against acetyl-DON:

Antibodies against deoxynivalenol-triacetate (Acetyl-DON or Ac-DON) were prepared by immunization of rabbits with the hemisucciate derivative of 7,8 dihydroxycalonectrin (DHC) conjugated to bovine serum albumin. Using tritiated Ac-DON as the testing ligand, antibody titers were demonstrated as early as 4 weeks after immunization. Useful antibody for radioimmunoassay of Ac-DON was obtained from the rabbits 7 weeks after immunization, with one booster injection. Competitive RIA revealed that the antibody was most specific to Ac-DON. The relative cross-reactivity of this antibody with Ac-DON, T-2 toxin tetra-acetate, 15 acetyl-DON and acetyl-T-2 toxin was found to be 1 (most), 0.003, 002, and 0.001, respectively. Practically no cross-reaction was found with DON, diacetoxyscirpenol, nivalenol and T-2 toxin. The detection limits for Ac-DON by RIA was around 0.1 ng/assay. The use of this antibody for quantitation and confirmation of DON in cereals is presently under study.

VI. STUDIES ON SAXITOXIN (STX):

(A) Characterization of antibody against saxitoxin (STX) and development of an indirect ELISA for STX:

In the last annual report, we described a procedure to produce antibody against STX. The antibody against STX was further characterized by a competitive indirect ELISA. The results showed that reduced STX and tetradotoxin did not cross-react with the antibody. Neo-STX reacted with the antibody but was 100 times less effective as compared with STX. Investigation also led to the application of the ELISA technique to the analysis of STX in clam and mussel. Preliminary

results showed that mussel extracts gave considerable interference in the assay at a concentration above 10 mg of sample per assay. The assay system appeared to tolerate about 50 mg of clam extracts in each analysis. Because more than 90% of STX is bound to the dark gland which is relatively easy to separate from whole meat, we have tested the effect of the dark gland matrix of mussel extracts on the ELISA of STX. The degree of interference caused by the dark gland matrix was found to be similar to the whole meat. This result suggested that for mussel, one can use the dark gland (approximately 1/10 of weight of whole meat) for ELISA of STX. Without any cleanup, we found that the detection limit of ELISA for STX in mussel (using dark gland) and clam was around 50-100 ppb.

(B) Efforts to improve antibody production and ELISA for STX:

(1) Improvement of ELISA protocols: Efforts for improving ELISA protocols continued. Amberlite CG-50 was used to remove the interference substances from the naturally contaminated scallops. Several ion-exchange filter papers were tested for their effectiveness of absorption of STX. After absorption of STX, these filter papers were subjected to an indirect ELISA. However, we found that the antibodies and other proteins also non-specifically absorbed on the filter paper. A high background color was observed. Other approches such as the use of a Sep-Pak treatment were also unsuccessful.

(2) Immunization of rabbits with conjugates by conjugating STX to BSA via glutaraldehyde (GA): Conjugation of STX to BSA was made in the presence of glutaradehyde instead of HCHO. However, the final preparation became a gel because intermolecular cross-linking of BSA occurred during the coupling reaction. This preparation was used in the immunization. Low antibody titers (100) were demonstrated in an indirect ELISA (plate coated with STX-GA-BSA) 4 weeks after initial injection and one boosterinjection.

(3) Attempts to produce a monoclonal antibody against STX: A total of 47 mice were immunized with either STX-HCHO-BSA or STX-HCHO-hemocyanin by intradermal, subcutaneous, and intrasplenic routes. Although most mice show antibody titers by an indirect ELISA screen test, only two mice produced antibody which was displaceable by free STX at a concentration of 250 ng/well after 3 booster injections over a period of two months.

VII. IMMUNOHISTOCHEMICAL STUDIES ON DAS:

A group of mice fed DAS at a dose of 10 mg/kg. The mice were sacrificed at different times after dosing. Different organs were collected and examined for pathological lesions as well as for immunohistochemical stains according to the procedures described previously (Publication I-1). Preliminary data showed that no significant lesions were found in the organs of the mice fed at this dosage. We will continue to evaluate the data for this study.

VIII. NEUTRALIZATION OF T-2 TOXIN TOXICITY WITH ANTI-T-2-ANTIBODY:

Passive aounization of CF-1 aice with rabbit anti-T-2 antiserua was carried out. In a preliaarily experiaent, the levels of circulating antibody and its persistence after passive iaaunizatica with 0.5 al of anti-T-2 antiserua (titer 5,000) without challenge with T-2 toxin was deterained. We found that antibody titer was detected at 3 hrs post-injection with the antiserua. Levels reaained constant fraa 24-96 hrs at a titer (RIA) approxiaately 10% of that injected. Detectable levels of antibody persisted through 144 hours post-injection. However, when challenged with T-2 toxin, no significanct difference between the passive iaaunized aice and the control aice on the course of T-2 toxicosis, as deterained by the LD_{50}, circulating leukocytes, and serua alkaline phosphatase, was observed. Using ^{3}H-T-2 toxin, we also did not find any significant difference on the distribution and clearance of T-2 toxin in the passive iaaunization group. Because only a saall aaout of antiserua was used in tne present study, the resul'.s indicate that a large aaount of high titer antiserua aay be necessary for the protection.

III. DISCUSSION

During the last two contract years, rapid progress in the area of iaaunoassays for trichothecene aycotoxins were aade in our laboratory. A nuaber of antibodies against trichothecene aycotoxins were produced in our laboratory during the last few years. The properties of these antibodies were studied in detail. Antibody against saae trichothecene aetabolites were also aade available. The cross-reactivities of several iaportant trichothecene antibodies with different trichothecenes are suaarized in Tables I and II. It is apparent that each antibody has its own specificity. Efforts to produce a generic antibody attempting to detect all types of trichothecenes were unsuccessful. However, as aore data are accuaulated, we found that the side chain of trichothecene groups played an iaportant role in eliciting antibody against this group of aycotoxins. Therefore, it is possible to produce antibodies against several types of aycotoxins within the trichothecene group through aodification of the side chains. In this regard, we have aade antibody against acetyl-DON and T-2 tetraol tetra-acetate. These antibodies will be useful for detection of DON as well as T-2 tetraol.

With the avialability of antibodies, studies to optiaize the conditions for RIA and ELISA of different toxins in different saaple aatrices should be continued. Because our efforts were focused on the developaent of different antibodies, only liaited experiaents were carried out for iaproving the RIA and ELISA in the present contract period. Aore efforts will be focused in this direction in the coaing year. To iaprove ELISA, we plan to test different enzyae preparations and also to use different aethods for conjugation of the toxins to the enzyaes. We also plan to spend aore efforts on STX research in the coaing year.

Studies on the use of RIA to aonitor the production of T-2 and DAS by different Fusaria species further proved the advantages of RIA over

other analytical methods. Our results also indicated that the ability of to produce toxins varied considerably with the species tested, as well as temperatures and substrates used. This information will be extremely useful for our understanding of the etiology of toxin production. Studies on the modification of T-2 metabolism by esterase inhibitors not only led to a new approach for the production of some major T-2 metabolites but also unveiled the possible impact of enviornmental agents on the T-2 toxicity.

IV. DELIVERABLES

The immunochemical reagents prepared and delivered to the USAMRRID since beginning of this contract are summarized in Table III.

V. LIST OF PUBLICATIONS

I. PUBLISHED:

1. Lee, S. C., Beery, J. T. and Chu, F. S. 1984. Immunoperoxidase localization of T-2 toxin. Toxicol. & Appl. Pharmacol.72:228-235.

2. Chu, F. S. 1984. Immunoassays for analysis of mycotoxins. J. Fd. Prot. 47:562-569.

3. Chu, F. S. 1984. Immunochemical studies on mycotoxins. In "Toxigenic Fungi-Their toxins and Health Hazard." ed. by H. Kurata and Y. Ueno, p.234. Kodansha (Tokyo) and Elsevier (Amsterdam-N.Y. & Tokyo).

4. Fan, T. S., Zhang, G. W. and Chu, F. S. 1984. An indirect ELISA for analysis of T-2 toxin in biological fluids. J. Fd. Prot. 47: 964-968.

5. Chu, F. S., Liang, M.Y. and Zhang, G.S. 1984. Production and characterization of antibody against diacetoxyscirpenol. Appl. Environ. Microbiol. 48: 777-780.

6. Chu, F. S., Zhang, G. S., Williams, M. D. and Jarvis, B. B. 1984. Production and characterization of antibody against deoxyverrucarol. Appl. Environ. Microbiol. 48:781-784.

7. Chu, F. S. and Fan, T. S. L. 1985. An indirect enzyme-linked immunosorbent assay for saxitoxin. J. Assoc. Off. Anal. Chem. 68:13-16.

8. Hunter, K. W., Jr., Briefield, A. A., Miller, M., Finkelman, F.D., and Chu, F. S. 1985. Preparation and characterization of monoclonal antibodies to the trichothecene mycotoxin T-2. Appl. Environ. Microbiol. 49: 168-172.

9. Wei, R. D. and Chu, F. S. 1985. Modification of metabolism of T-2 toxin by esterase inhibitors. Appl. Environ. Microbiol. 50: 115-119.

II. MANUSCRIPTS SUBMITTED OR PREPARED

1. Zhang, G. S., Schubring, S. L. and Chu, F. S. 1985. An improved method for the production of antibody against T-2 toxin and diacetoxyscirpenol. Submitted to Appl. Environ. Microbiol.

2. Wei, R. D., Bischoff, M. B. and Chu, F. S. 1985. Production and characterization of antibodies against 3'-OH-T-2 toxin. Submitted to J. Fd. Prot.

3. Zhang, G. S., Li, S. M. and Chu, F. S. 1985. Production and characterization of antibody against deoxynivalenol triacetate. Submitted to J. Fd. Prot.

4. Fan, T. S. L., Zhang, G. S. and Chu, F. S. 1985. Production and characterization of antibodies against HT-2 toxin and T-2 tetraol tetra-acetate. In preparation.

TABLE 1 RELATIVE CROSS-REACTIVITY OF ANTIBODIES AGAINST T-2 TOXIN WITH DIFFERENT T-2 TOXIN ANALOGS

TOXIN ANALOGS	RELATIVE CROSS-REACTIVITY				
	HS	a-CHO	b-CHO	3'-OH-HS	CHO-HT-2
Ac-T-2	1	4	3	3	30
T-2	1	1	1	4	4
ISO-T-2	2	-	-	30	10
HT-2	6	5	2	45	1
T-2 triol	44	200	488	>2500	850
T-2 tetraol	1428	2608	-	>2500	>1613
3'-OH-Ac-T-2	50	-	-	5	1230
3'-OH-T-2	200	434	-	1	680
3'-OH-T-2HS	-	-	-	15	>1613
3'-OH-HT-2	-	-	-	175	400
NEOS	453	-	-	-	-
DAS, DON	>2857	>4300	-	>2500	>1613

TABLE 2 RELATIVE CROSS-REACTIVITY OF ANTIBODIES AGAINST DAS AND OTHERS WITH DIFFERENT TRICHOTHECENES

TRICHOTHECENES	RELATIVE CROSS-REACTIVITY			
	DAS-HS	b-CHO-DAS	DOVE-HS	AC-DON
Ac-DON	-	76	-	1
15-Ac-DON	-	-	-	470
Ac-T-2	-	147	-	900
DAS	1	1	31250	>1000
4-MAS	87	4	-	-
15-MAS	200	5	-	-
DON	>1333	>2000	>31250	>1000
DOVE	>1333	-	1	-
NEOS	>1333	266	-	-
NIV	2520	-	-	>1000
T-2	>1333	107	>31250	>1000
T-2-4-Ac	-	-	-	380
Verrucarol	-	-	3343	-
Verrucarin A	-	-	29687	-

TABLE 3. IMMUNOCHEMICALS DELIVERED BETWEEN AUG. 1, 1983 TO AUG. 31, 1985.

REAGENT NAME	AMOUNT
H-DAS	1.0 MCi
H-DOVE	1.0 MCi
H-VERRUCARIN A	2.0 MCi
HT-2	10.0 MG
3'-OH-T-2	10.0 MG
3'-OH-HT-2	1.0 MG
DAS-HS-BSA	48.5 MG
DOVE-HS-BSA	18.0 MG
STX-BSA	22.5 MG
T2-HS-BSA	30.3 MG
T-2 HS-BSA	5.0 MG
T-2-HS-POLYLYSINE	25.0 MG
T-2-HS-PEROXIDASE	6.0 MG
DAS ANTIBODY	41.0 ML
DOVE ANTIBODY	13.0 ML
T-2 ANTIBODY	42.0 ML

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